TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. § 371		Attorney's Docket Number 045636-5055 U.S. Application No. Unassigned
International Application. No.	International Filing Date	Priority Date Claimed
PCT/FR00/02565	September 15, 2000	September 17, 1999

Title of Invention: METHOD FOR GENETIC MODIFICATION OF LACTOBACILLUS DELBRUECKII

Applicants For EO/EO/US: Pascale SERROR, Golnar ILAMI-NESPOULOUS, Maarten VAN DE GUCHTE, Christian CHERVAUX, Christophe FREMAUX, Laurent BENBADIS and Emmanuelle MAGUIN

Applicants herewith submit to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a FIRST submission of items concerning a filing under 35 U.S.C. § 371.
2. ☐ This is a SECOND or SUBSEQUENT submission of items concerning a filing under 35 U.S.C. § 371.
3. ☐ This express request to begin national examination procedures (35 U.S.C. § 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. § 371(b) and PCT Articles 22 and 39(l).
4. ☒ A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.
5. ☒ A copy of the International Application as filed (35 U.S.C. § 371(c)(2))
   a. ☐ is transmitted herewith (required only if not transmitted by the International Bureau).
   b. ☒ has been transmitted by the International Bureau.
   c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☒ A translation of the International Application into English (35 U.S.C. § 371(c)(2)).
7. ☒ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. § 371(c)(3)).
   a. ☐ are transmitted herewith (required only if not transmitted by the International Bureau).
   b. ☐ have been transmitted by the International Bureau.
   c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
   d. ☒ have not been made and will not be made.
8. ☐ A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. § 371(c)(3)).
9. ☐ An oath or declaration of the inventors (35 U.S.C. § 371(c)(4)).
10. ☐ A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. § 371(c)(5)).

Items 11. to 14. below concern other document(s) or information included:

11. ☐ An Information Disclosure Statement under 37 C.F.R. § 1.97 and § 1.98.
12. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 C.F.R. § 3.28 and § 3.31 is included.
13. ☒ A FIRST preliminary amendment.
    ☐ A SECOND or SUBSEQUENT preliminary amendment.
14. ☒ Other items or information:
    a. WO 01/21819 cover sheet including English language abstract
    b.
    c.
    d.
    e.
    f.

10/088293

JC10 Rec'd PCT/PTO 15 MAR 2002

U.S. APPLICATION NO.	INTERNATIONAL APPLICATION NO.	ATTORNEY DOCKET NUMBER
Unassigned	PCT/FR00/02565	045636-5055

15. ☒ The following fees are submitted:

Basic National Fee (37 C.F.R. § 1.492(a)(1)-(5)):

Search Report has been prepared by the EPO or JPO.....$890.00
International preliminary examination fee paid to
USPTO (37 C.F.R. § 1.482).....$710.00
No international preliminary examination fee paid to
USPTO (37 C.F.R. § 1.482) but international search fee
paid to USPTO (37 C.F.R. § 1.445(a)(2)).....$740.00
Neither international preliminary examination fee
(37 C.F.R. § 1.482) nor international search fee
(37 C.F.R. § 1.445(a)(2)) paid to USPTO.....$1,040.00
International preliminary examination fee paid to USPTO
(37 C.F.R. § 1.482) and all claims satisfied provisions
of PCT Article 33(2)-(4).....$100.00

ENTER APPROPRIATE BASIC FEE AMOUNT = $890.00

Surcharge of $130.00 for furnishing the oath or declaration later than
☐ **20** ☒ **30** months from the earliest claimed priority date
(37 C.F.R. § 1.492(e)). $

Claims	Number Filed	Number Extra	Rate	
Total Claims	10 - 20 =	0	X $18.00	$
Independent Claims	4 - 3 =	1	X $84.00	$ 84.00
Multiple dependent claim(s) (if applicable)			+ $280.00	$
		TOTAL OF ABOVE CALCULATIONS		$ 974.00
		Reduction by ½ for filing by small entity, if applicable. Verified Small Entity statement must also be filed. (Note 37 C.F.R. §§ 1.9, 1.27, 1.28)		-$
		SUBTOTAL =		**$ 974.00**
Processing fee of $130.00 for furnishing the English translation later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 C.F.R. § 1.492(f)).				+$
		TOTAL NATIONAL FEE =		**$974.00**
Fee for recording the enclosed assignment (37 C.F.R. § 1.21(h)). The Assignment must be accompanied by an appropriate cover sheet (37 C.F.R. §§ 3.230, 3.31). $40.00 per property				$
		TOTAL FEES ENCLOSED =		**$**
		Amount to be refunded		$
		Amount to be charged		$

a. ☐ A check in the amount of $_____ to cover the above fees is enclosed.
b. ☒ Please charge my Deposit Account No. 50-0310 in the amount of **$974.00**
c. ☒ **Except** for issue fees payable under 37 C.F.R. § 1.130, the Commissioner is hereby authorized by this paper to charge any additional fees during the entire pendency of this application including fees due under 37 C.F.R. § 1.16 and § 1.17 which may be required, or credit any overpayment to Deposit Account No. 50-0310.

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Submitted: March 15, 2002

Attorney Docket No. 045636-5055

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of : Pascale SERROR et al.	
U.S. Application No.: To Be Assigned	Group Art Unit: Unassigned
Date of National Stage Entry : March 15, 2002	Examiner: Unassigned
Based on PCT/FR00/02565 Filed : September 15, 2000	
For: METHOD FOR GENETIC MODIFICATION OF LACTOBACILLUS DELBRUECKII	

Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

PRELIMINARY AMENDMENT

Prior to the examination of the above-identified application on the merits, please amend the application, without prejudice, as follows:

IN THE CLAIMS:

Cancel claims 1-10.

Add the following claims 11-20:

11. A method of introducing a modification of the genetic material of a bacterium comprising introducing a plasmid comprising the pIP501 theta replication system, or a related replication system, as a thermosensitive conditional vector for integration into a bacterium of the species *Lactobacillus delbrueckii.*

12. A method for modifying the genetic information carried by the chromosome of a bacterium of the species *Lactobacillus delbrueckii* comprising:

a) constructing an integrative plasmid by inserting at least one DNA sequence capable of integrating into the bacterial chromosome into a conditional vector comprising the pIP501 theta replication system, or a related replication system, wherein the integrative plasmid also carries at least one selection marker;

b) introducing the plasmid into a *Lactobacillus delbrueckii* bacterium and multiplying the resultant bacterium under conditions which permit the replication and maintenance of the plasmid in stable form;

c) multiplying the bacteria under conditions which prevent the replication and/or maintenance of the plasmid in stable form.

13. The method as claimed in claim 12, further comprising recovering the bacteria expressing at least one selection marker originating from the plasmid..

14. The method of claim 12 wherein the DNA sequence capable of inserting into the bacterial chromosome is a sequence homologous to a portion of the chromosome into which it is desired to introduce a modification.

15. The method of claim 12 wherein the DNA sequences capable of inserting into the bacterial chromosome is a transposable sequence.

16. The method of claim 15 wherein the transposable sequence is an insertion sequence.

17. The method of claim 16, wherein the insertion sequence is chosen from IS1223 of *Lactobacillus johnsonii* and IS1201 of *Lactobacillus helveticus*.

18. A method of modifying the chromosome of *Lactobacillus delbrueckii* comprising introducing an insertion sequence chosen from IS1223 from *Lactobacillus johnsonii* and IS1201 of *Lactobacillus helveticus* into the nucleus of *Lactobacillus delbrueckii* cells.

19. An integrative plasmid comprising one of the sequences IS1223 or IS1201 and either:

a) the pVE6002 replication system, or

b) the pIP501 replicon theta replication system or a related replication system.

20. The integrative plasmid of claim 19, wherein the plasmid is chosen from the group consisting of the pVI49 plasmid and the pVI52 plasmid, deposited with the CNCM on September 17, 1999, under the respective numbers I-2317 and I-22318.

REMARKS

The changes to the claims requested above have been made so as to eliminate multiple claim dependencies and to present claim language more conventional for practice in the United States.

These changes do not introduce new matter, nor do they alter the subject matter presented and examined in the corresponding International Application.

If there are any additional fees due in connection with the filing of this Preliminary Amendment, please charge the fees to our Deposit Account No. 50-0310.

Respectfully submitted,

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Dated: March 15, 2002
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1-WA/1765930.1

METHOD FOR GENETIC MODIFICATION OF LACTOBACILLUS DELBRUECKII

The present invention relates to the genetic modification of *Lactobacillus delbrueckii*.

Lactic acid bacteria are used a great deal in the agrofoods industry, particularly for manufacturing diverse fermented products; in addition, their innocuity has made their use recommended for the production, by genetic engineering, of diverse substances intended in particular for therapeutic use.

The genetic modification of lactic acid bacteria makes it possible to adjust their characteristics depending on the use envisaged, whether by the introduction and expression of foreign DNA, or by the overexpression or, on the contrary, the inactivation of genes naturally present in said bacteria. For a modification to be stable, it must be integrated into the bacterial chromosome. With this aim, the desired modification is inserted into a nonreplicative plasmid carrying a selection marker. The vector thus obtained is introduced into the bacteria; the bacteria expressing the selection marker, which are those which have integrated the vector into their chromosome, are then recovered.

The modification results from 2 events which occur at low frequency: 1) the introduction of the plasmid into the bacteria; 2) the integration of said plasmid into the chromosome. The modification rate which may be anticipated is the product of the probabilities of these two events; it is therefore very low, especially in the case of bacteria belonging to species which are "refractory" to transformation, which is the case for many species of lactic acid bacteria. Among these, mention will be made in particular of the species

Lactobacillus delbrueckii, which in particular comprises the subspecies *Lb. delbrueckii* subsp. *bulgaricus*, *Lb. delbrueckii* subsp. *lactis*, and *Lb. delbrueckii* subsp. *delbrueckii*.

A single example of a vector which allows integration into the chromosome of *Lb. delbrueckii* is described in the prior art; it is the conjugative plasmid pAMβ1, described as being nonreplicative in *Lb delbrueckii*. Application EP 0603416 in the name of MEIJI MILK PRODUCTS CO LTD reports the use of this plasmid to integrate a modification into the chromosome of *Lb. delbrueckii*: a selection marker (erythromycin resistance) was inserted into a fragment homologous to a region of the chromosome of *Lb. delbrueckii*, and the entire combination was introduced into the pAMβ1 plasmid. The integrative plasmid thus constructed was amplified in *Lactococcus lactis*, and then transferred by conjugation into *Lb. delbrueckii*; the trans-conjugants/integrants are then selected on the basis of the erythromycin resistance resulting from the integration, by homologous recombination, of the insert from the plasmid into the chromosomal DNA.

Since these modifications are obtained at very low frequency, it is desirable to have other integrative vectors which are suitable for *Lb. delbrueckii* and facilitate the production of the modified strains and the selection of the integrants.

In order to increase the modification rate and facilitate the screening of the modified bacteria, the use of conditional plasmids as vectors has been proposed. The term "conditional" is used herein to describe a vector, for example a plasmid, for which at least one of the functions of replication, partition or stability is active only under certain conditions (for example of temperature, of pH, of inorganic salt concentration, of presence in the culture medium of a

particular compound, etc.), either due to the fact that one or more of the proteins involved in this function comprise(s) one or more mutation(s) making the activity conditional, or due to the fact that one or more of the proteins involved in this function is (are) placed under the control of an inducible promoter.

BISWAS et al., J. Bacteriol., 175, 3628-3635, (1993); MAGUIN et al., J. Bacteriol., 178, 931-935, (1996) and PCT application WO 93/18164 thus describe integrative vectors which comprise the pVE6002 mutant replicon replication system (CNCM I-1179), also named pG$^+$host, which is a thermosensitive (Ts) derivative of the plasmid pWV01 due to a mutation in the sequence encoding the RepA protein.

The modification may thus be carried out in two steps: in the first, the plasmid is introduced into the bacteria, at permissive temperature, which allows a first selection of those in which it has installed itself; in the second, these bacteria are cultured at a temperature which is nonpermissive for the replication of the plasmid, which makes it possible to select those in which the sequences introduced are integrated into the chromosome.

PCT application WO 93/18164 describes the use of these plasmids in diverse species of lactic acid bacteria, including *Lb. delbrueckii* subsp. *bulgaricus*.

In pursuing their investigations, the inventors have presently noted that other plasmids used in lactic acid bacteria are capable of replicating in *Lb. delbrueckii* at temperatures possibly reaching approximately 37°C, which allow the growth of this species, but are thermosensitive at higher temperatures, generally from 42°C, which is the optimum temperature for growth of this species.

They are in particular derivatives of the plasmid pIP501; it has been shown that this plasmid may be transferred by conjugation into *Lb. delbrueckii* subsp. *bulgaricus* and replicate therein [LANGELLA and CHOPIN, FEMS Microbiol. Lett., 60, 149-152, (1989)]. It has been described as thermosensitive in *B. subtilis* and several lactobacilli, including *L. helveticus* [BHOWMICK and STEELE, J. Gen. Microbiol., 139, 1433-1439, (1993)] *L. plantarum* [RIXON et al., FEMS Microbiol. Lett., 71, 105-110, (1990)] and *L. acidophilus* [LUCHANSKI et al., Mol. Microbiol., 2, 637-646, (1988)], but not in *Lb. delbrueckii*. The inventors have established that pIP501 derivatives are thermosensitive in *Lb. delbrueckii*; they can in fact replicate and maintain themselves at approximately 35-37°C in this bacterial species, but their replication and/or their maintenance in stable form are inhibited from approximately 42°C.

These pIP501 derivatives have been used by the inventors to transform *Lb. delbrueckii* and have made it possible, at nonpermissive temperature, to obtain integrants.

Consequently, a subject of the present invention is the use of a plasmid comprising the pIP501 theta replication system, or a related replication system, as a thermosensitive conditional vector for integration which makes it possible to introduce a modification of the genetic information of a bacterium of the species *Lb. delbrueckii*.

This modification is introduced into the chromosome via one or more transposition and/or homologous recombination events.

The expression "replication system related to the pIP501 theta replication system" defines herein any theta replication system which has the functional characteristics, and in particular the

thermosensitivity in *Lb. delbrueckii*, of pIP501. This encompasses in particular any theta replication system comprising:

- an origin of replication, *ori*, having at least 80% identity, preferably at least 85% identity and advantageously at least 90% identity, at the level of the nucleotide sequence, with the origin of replication, *ori*, of pIP501 and/or;
- a sequence encoding a *rep* protein, the peptide sequence of which has at least 85% identity, preferably at least 90% identity, and advantageously at least 95% identity, with that of the *repR* protein of pIP501.

The sequence of the origin of replication, ori, of pIP501 is in particular described by BRANTL and BEHNKE [Molecular Microbiology, 6, 3501-3510 (1992)]; the sequence encoding the *repR* of pIP501 is in particular identified in the complete sequence of pGB3631 (derived from pIP501), published by BRANTL et al., [Gene, 142, 155-156, (1994)], and accessible under the number X72021.

The percentage identity of a sequence with a reference sequence is defined herein as the percentage of residues of this sequence which are identical to those of the reference sequence over an alignment of the 2 sequences which ensures that the positions of the residues correspond to a maximum.

A subject of the present invention is in particular a process for modifying the genetic information carried by the chromosome of a bacterium of the species *Lb. delbrueckii*, characterized in that it comprises:

a) constructing an integrative plasmid by inserting at least one DNA sequence, capable of integrating into the bacterial chromosome, into a conditional vector comprising the pIP501 theta replication system, or a related replication system, said

vector also carrying at least one selection marker;

b) introducing the plasmid into said bacterium and multiplying this bacterium, under conditions permissive for the replication and maintenance in stable form of said plasmid;

c) multiplying the bacteria expressing at least one selection marker from the plasmid at the end of step b), under conditions nonpermissive for the replication and/or maintenance in stable form of the plasmid; and, optionally,

d) recovering the bacteria expressing at least one selection marker originating from the plasmid, at the end of step c).

According to a variant of the process in accordance with the invention, the step of multiplying the bacterium under conditions permissive for the replication and maintenance of the plasmid in stable form may be omitted; in this case, after introducing the plasmid into the bacterium, the multiplication thereof is carried out directly under conditions nonpermissive for the replication and/or maintenance in stable form of the plasmid.

Advantageously, it is possible, after having obtained bacteria carrying the desired modification, to remove the sequences originating from the vector used in step a), in cases in which it is not desirable to keep these sequences, for example in the case of bacteria intended for industrial use, in particular for the preparation of foodstuffs.

In this case, the process in accordance with the invention also comprises the following steps:

e) excising the sequences originating from the vector, by multiplying the bacteria expressing, at the end of step c), at least one selection marker originating from the plasmid and, advantageously,

f) removing the excised DNA by multiplying the bacteria obtained in step e), under conditions nonpermissive for the replication and maintenance of the vector in stable plasmid form.

Conditional vectors comprising the pIP501 theta replication system, or a related system, used in *Lb. delbrueckii* for carrying out the present invention are thermosensitive plasmids which are capable of replicating and of maintaining themselves at 35-37°C in this bacterial species, and the replication and/or maintenance of which in stable form are inhibited from approximately 42°C.

These plasmids also carry at least one selection marker, i.e. a gene which can be expressed in *Lb. delbrueckii* and the expression of which confers on the bacteria harboring it a distinctive phenotype which allows them to be selected. It may, for example, be a resistance marker which confers a characteristic of resistance to a substance usually toxic for the bacterium, for example an antibiotic, or an auxotrophy marker which confers the ability to grow in the absence of a nutrient usually essential to the bacterium. The bacteria expressing these markers are, for example, easily selected by virtue of their survival on a selective medium, i.e. in the presence of said toxic substance or in the absence of said nutrient. Thus, at the end of step b) of the process above, the bacteria in which the plasmid is present may be selected on the basis of the expression of a selection marker originating from the plasmid. Similarly, at the end of step c), the bacteria in which the DNA of the plasmid has been integrated into the chromosome may be selected on the basis of the expression of a selection marker originating from the plasmid constructed in step a).

Another possibility for selection at the end of step c) consists in using a property of the strain which ensues

from the integration of the plasmid and/or from the excision of the vector sequences. For example, use may be made of a conditional plasmid comprising a selection marker M (for example a resistance marker) and a DNA sequence capable of integrating by homologous recombination into a bacterial gene X, said gene X being essential under certain conditions for the bacterium (for example a gene essential for the growth thereof on a given medium), the integration of the plasmid inactivating the gene X, and the excision of the sequences of the vector restoring its activity. In this case, the bacteria in which the plasmid DNA has been integrated may be selected on the basis of the expression of the selection marker M, under conditions in which the gene X is not essential, and the bacteria in which the sequences of the integrated vector have been excised may be selected, under conditions in which the gene X is essential, on the basis of the restoration of its activity. In addition, this method of selection also makes it possible to select, in a single step, bacteria in which the integration of the plasmid DNA and the excision of the sequences of the vector have taken place; in this case, the bacteria are directly cultured under conditions in which the gene X is essential, and which are also selective for the marker M. The bacteria selected will be those in which the integration of the plasmid and the excision of the sequences of the vector (including the marker M) have restored the activity of the gene X.

DNA sequences capable of integrating into the chromosome of a bacterium of the species *Lb. delbrueckii* in particular comprise:

- sequences chosen on the basis of their homology with a portion of the chromosome into which it is desired to introduce a modification, i.e. exhibiting a homology with this portion of the chromosome which is sufficient to be able to recombine with it;

- transposable sequences, in particular transposons or insertion sequences (IS); such sequences may insert into the bacterial chromosome randomly or with a certain specificity.

The modifications intended to be integrated into the bacterial chromosome may be introduced by the simple integration of these sequences, this integration possibly, for example, leading to the inactivation of a gene within which it occurs. It is also possible to use these sequences to integrate, into the bacterial chromosome, a DNA fragment, in particular a gene of interest of heterologous origin, or a DNA fragment from *Lb. delbrueckii*, modified beforehand.

In the case of the integration by homologous recombination, for example, it is possible to introduce, into a sequence identical to that of the region of the chromosome intended to be modified, modifications by insertion, deletion or substitution which may range from a single nucleotide to several thousands of nucleotides. The sequence thus modified is inserted in step a) of the process according to the invention, into a vector which is conditional in *Lb. delbrueckii*, and which comprises the pIP501 theta replication system, or a related system, as defined above. The integration takes place by recombination (simple crossing-over) between the cloned chromosomal DNA fragment and the homologous region of the bacterial chromosome. Since this recombination event creates duplications of the region of homology, on both sides of the vector sequences, the structure integrated into the chromosome consists of the vector sequences bordered on both sides by a copy of the region of homology, as shown in figure 1.

The use of sequences which can integrate by homologous recombination in particular makes it possible:

- to inactivate one (or more) bacterial gene(s);
- to modify the expression of the genes and/or the activity of the products encoded by these genes;
- to introduce, in a stable manner, new functions into the chromosome;
- to study and to use the expression of the genes *in situ*;
- to mutate the chromosome by integration via random chromosomal fragments;
- to insert, in a stable manner, sequences intended to label the strains. These sequences will subsequently be used as tracers;
- to introduce several sequential modifications into the same strain.

In the case of the integration via a transposable sequence, the transposable sequence will be inserted into the plasmid; the integration into the chromosome makes it possible to produce the modification, to thus obtain mutants exhibiting advantageous characteristics, and to characterize more easily the mutated gene(s). The structure transposed into the chromosome may consist of the vector sequences bordered on both sides by a copy of the transposable sequence.

These transposable sequences may originate from bacteria belonging to the species *Lb. delbrueckii*, or originate from other bacterial species, in particular other lactic acid bacteria. They may be transposons or insertion sequences.

Transposable sequences which are functional in *Lb. delbrueckii* may be identified by inserting a transposable sequence to be tested (transposon or insertion sequence) into a vector which replicates conditionally in *Lb. delbrueckii* and which comprises the pIP501 theta replication system, or a related system, as defined above, by carrying out steps b) and c) of the process in accordance with the invention and

searching for the presence of transposants at the end of these steps.

The inventors have thus demonstrated 2 insertion sequences which are functional in *Lb. delbrueckii*. A subject of the present invention is also the use of either of these sequences for modifying the chromosome of *Lb. delbrueckii*.

They are the insertion sequence named IS*1233*, previously demonstrated by WALKER et al. (J. Bacteriol., 176, 5330-5340, 1994) in *Lb. johnsonii*, and the insertion sequence named IS*1201*, previously demonstrated by TAILLIEZ et al. (Gene, 145, 75-79, 1994) in *Lb. helveticus*.

A subject of the present invention is also any integrative plasmid resulting from the insertion of one of these 2 sequences into a vector which replicates conditionally in *Lb. delbrueckii*, and in particular into a vector comprising the pVE6002 replication system, such as those described in PCT application WO 93/18164, or into a vector comprising the pIP501 theta replication system, or a related system, as defined above.

According to a preferred embodiment of the present invention, said vector is a nonconjugative vector.

Integrative plasmids in accordance with the invention are in particular illustrated by the pVI49 plasmid and the pVI52 plasmid. The pVI49 plasmid harbored by the *E. coli* strain VI209, and the pVI52 plasmid harbored by the *E. coli* strain VI217, were deposited, according to the Treaty of Budapest, on September 17, 1999, under the respective numbers I-2317 and I-2318, with the CNCM (Collection Nationale de Cultures de Microorganismes [National Collection of Microorganism Cultures]), 25 rue du Docteur Roux, Paris.

In the two scenarios: integration by homologous recombination or by transposition of a mobile element, the sequences originating from the vector may be excised, during step e) of the process in accordance with the invention, by recombination between the homologous sequences flanking the vector sequences.

For example, in the case of the integration by homologous recombination, a second recombination event (double crossing-over) may take place between the duplicated homologous regions on both sides of the vector sequences. This event leads to the excision of the vector sequences and makes it possible, for a fraction of the clones, to substitute the wild-type chromosomal form with the modified plasmid form, as shown in figure 1.

Legend for figure 1:

A, B: chromosomal DNA cloned into the vector;
▲: modification;
CR: conditional replicon;
M: selection marker;
P: permissive conditions;
NP: nonpermissive conditions.

For example, in the case of the integration using certain transposable sequences, these sequences also constitute, on both sides of the vector sequences, homologous regions which promote recombination events which lead to the excision of the vector sequences and of one of the copies of the IS, the other remaining in the bacterial chromosome at the transposition site.

Figure 2 represents the excision of the sequences of a vector by homologous recombination between IS.

Legend for figure 2:

I: insertion sequence;
M: selection marker;

NP: nonpermissive conditions.

The bacteria selected at nonpermissive temperature during step c) (>42°C) are cultured without any selection pressure so as to allow recombination between the homologous regions flanking the vector sequences.

After removal of the excised sequences, in accordance with step f) of the process in accordance with the invention, the following are thus obtained:

- in the case of a plasmid comprising a sequence capable of integrating by homologous recombination, a bacterium which differs from the host bacterium of origin by the presence, in its chromosome, of the modification introduced by this sequence;
- in the case of a plasmid comprising a transposable sequence, a bacterium which differs from the host bacterium of origin by the presence, in its chromosome, of a copy of said transposable sequence.

The present invention will be more fully understood with the aid of the further description which follows, which refers to nonlimiting examples of the production of integrative plasmids and of their use in *Lb. delbrueckii*.

EXAMPLE 1: SELECTION OF PLASMIDS WHICH ARE THERMOSENSITIVE IN *Lb. delbrueckii*

Production of plasmids

An *L. bulgaricus* - *E. coli* shuttle plasmid consisting of pGB3631 (a sequence derivative of pIP501, (BRANTL et al., Gene, 142, 155-156, (1994)]) and of the pSKII origin of *E. coli* was constructed. This plasmid, named pVI1055, is represented in figure 3. It carries an *ery* gene for erythromycin (Ery) resistance.

Another pIP501 derivative, the plasmid pGB305Δ [LE CHATELIER et al., Plasmid, 29, 50-61, (1995))] was also tested.

Demonstration of the thermosensitivity in *Lb. delbrueckii*

Transformation of bacteria

Bacteria of the *Lb. delbrueckii* subsp. *bulgaricus* strain ATCC 11842 or of the strain VI104 deposited according to the Treaty of Budapest, on September 17, 1999, under the number I-2316, with the CNCM (Collection Nationale de Cultures de Microorganismes [National Collection of Microorganism Cultures]), 25 rue du Docteur Roux, Paris, are cultured in MRS medium containing 0.1% glycine (DIFCO), until the beginning of the stationary phase. They are then centrifuged and washed in electroporation buffer (0.4 M sucrose, 1 mM $MgCl_2$, 5 mM KH_2PO_4, pH 6.0) and suspended in this same buffer at a concentration corresponding to an OD_{600} of approximately 50. The suspension is incubated for 20 minutes at 45°C and then cooled on ice. An 80 µl aliquot of the suspension is mixed with the plasmid DNA (~1.5 µg) and the mixture is transferred into a 0.2 cm electroporation cuvette. The electroporation is performed at 1 kV, 800 Ω, and 25 µF.

Immediately after electroporation, the mixture is diluted in 2 ml of expression medium (0.2 M sucrose, 5% powdered skimmed milk, 0.1% yeast extract, 1% casamino acids, 25 mM $MgCl_2$). After incubation for 3 h at 37°C in this medium, the cells are plated out onto dishes containing selective medium (MRS agar with 10 µg/ml of erythromycin); the dishes are incubated in anaerobic jar at 37°C for 48 h, and the erythromycin-resistant colonies are selected.

Thermosensitivity of pIP501 derivatives (theta replication)

The thermosensitivity of pGB305Δ and of pVI1055 was first evaluated using *Lb. delbrueckii* (ATCC 11842 or VI104) transformation assays according to the protocol described above, with plating out onto selective medium at 37°C and 42°C. For both plasmids, transformants are obtained at 37°C but not at 42°C.

The thermosensitivity of pVI1055 was then confirmed by comparing its stability at 37°C and at 44°C. A culture in MRS/Ery (10 µg/ml) at 37°C was diluted in MRS without Ery, and then incubated in parallel at 37°C and at 44°C. Samples are taken at various times and the cells are plated out after dilution on MRS and MRS/Ery, in order to determine the proportion of cells having lost the plasmid.

At 37°C, approximately 30% of the cells have lost the pVI1055 plasmid after 48 hours. At 44°C, the stability of pVI1055 is clearly affected, since ~100% of the cells have lost the plasmid after 48 hours.

EXAMPLE 2: DEMONSTRATION OF THE TRANSPOSITION ACTIVITY OF INSERTION SEQUENCES (IS) IN *Lb. delbrueckii*

Insertion sequences isolated from lactic acid bacteria were cloned into pVI1055. At a temperature permissive for the replication of the plasmid, the multiplication of a transformant generates a bacterial population containing the plasmid. The IS present on the plasmid in the bacterial population may transpose into the chromosome. In the event of transposition of the IS, the transposed structure present in the chromosome may correspond to the plasmid vector bordered, on both sides, by a copy of the IS, as shown in figure 4. At nonpermissive temperature (44°C), the plasmids containing the IS lead to the production of Ery^R clones

with a frequency greater than that observed for pVI1055, the plasmid without IS. In the case of the production of Ery^R clones, structural analysis of the chromosomal DNA by Southern Blot with a probe corresponding to the IS tested makes it possible to verify that the integration indeed results from a transposition event.

Demonstration of the transposition:

The IS *1223* (WALKER and KLAENHAMMER, 1994, abovementioned reference) and *1201* (TAILLEZ *et al.*, 1994, abovementioned reference) were cloned into the pVI1055 plasmid, generating, respectively, the plasmids pVI48, pVI49 (corresponding to the 2 orientations of IS*1223*) and pVI52.

The properties of these 2 IS and the nature of the plasmids obtained are indicated in table 1 below.

TABLE 1

IS	Origin	Size[a] (bp)	Final vectors[b]
1223	*L. johnsonii*	1502	pVI48/pVI49
1201	*L. helveticus*	1387	pVI52

a: size corresponding to the IS bordered by the direct repeats (DR)
b: when two plasmids are given, this indicates that the IS was cloned in both orientations

In order to search for the transposition activity, the strains containing the plasmids carrying the IS to be tested were cultured at 37°C in MRS/Ery (10 µg/ml). The cells are then diluted in MRS and incubated at 44°C. Samples were taken at various times and plated out on MRS dishes (to determine the viable cell count) and on MRS/Ery (Ery^R cell count).

Results:

IS*1223* (pVI48/pVI49): the pVI48 or pVI49 plasmid was introduced into VI104. In the presence of IS*1223*, the frequency of Ery^R cells is higher than with pVI1055 alone. After digestion, the chromosomal DNA of the Ery^R

clones obtained are hybridized with IS*1223*. In all cases, the Ery^R clones are the result of transposition events.

IS*1201* (pVI52): the pVI52 plasmid was introduced into VI104. The frequency of integration of pVI52 is higher than that of pVI1055 alone. All the Ery^R clones analyzed by Southern are the result of transposition.

These results illustrate the transposition activity of IS 1223 and 1201 in *Lb. delbrueckii*.

CLAIMS

1. The use of a plasmid comprising the pIP501 theta replication system, or a related replication system, as a thermosensitive conditional vector for integration which makes it possible to introduce a modification of the genetic information of a bacterium of the species *Lb. delbrueckii*.

2. A process for modifying the genetic information carried by the chromosome of a bacterium of the species *Lb. delbrueckii*, characterized in that it comprises:

a) constructing an integrative plasmid by inserting at least one DNA sequence, capable of integrating into the bacterial chromosome, into a conditional vector comprising the pIP501 theta replication system, or a related replication system, said integrative plasmid also carrying at least one selection marker;

b) introducing the plasmid into said bacterium and multiplying this bacterium, under conditions permissive for the replication and maintenance in stable form of said plasmid;

c) multiplying the bacteria expressing at least one selection marker from the plasmid at the end of step b), under conditions nonpermissive for the replication and/or maintenance in stable form of said plasmid; and, optionally,

d) recovering the bacteria expressing at least one selection marker originating from the plasmid, at the end of step c).

3. The process as claimed in claim 2, characterized in that it also comprises:

e) excising the sequences originating from the vector, by multiplying the bacteria expressing,

at the end of step c), at least one selection marker originating from the plasmid and, advantageously,

f) removing the excised DNA by multiplying the bacteria obtained in step e), under conditions nonpermissive for the replication and maintenance of said vector in stable plasmid form.

4. The process as claimed in either of claims 2 and 3, characterized in that the DNA sequence capable of inserting into the bacterial chromosome is a sequence homologous to a portion of the chromosome into which it is desired to introduce a modification.

5. The process as claimed in either of claims 2 and 3, characterized in that the DNA sequence capable of inserting into the bacterial chromosome is a transposable sequence.

6. The process as claimed in claim 5, characterized in that said transposable sequence is an insertion sequence.

7. The process as claimed in claim 6, characterized in that said insertion sequence is chosen from IS*1223* of *Lb. johnsonii* and IS*1201* of *Lb. helveticus*.

8. The use of an insertion sequence chosen from IS*1223* from *Lb. johnsonii* and IS*1201* of *Lb. helveticus*, for modifying the chromosome of *Lb. delbrueckii*.

9. An integrative plasmid for carrying out a process as claimed in any one of claims 1 to 6, characterized in that it results from the insertion of one of the sequences IS*1223* or IS*1201*

into a plasmid chosen from:

- the plasmids comprising the pVE6002 replication system;
- the plasmids comprising the pIP501 replicon theta replication system, or a related replication system.

10. The integrative plasmid as claimed in claim 8, chosen from the group consisting of the pVI49 plasmid and the pVI52 plasmid, deposited with the CNCM on September 17, 1999, under the respective numbers I-2317 and I-2318.

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En ce qui concerne les codes à deux lettres et autres abréviations, se référer aux "Notes explicatives relatives aux codes et abréviations" figurant au début de chaque numéro ordinaire de la Gazette du PCT.

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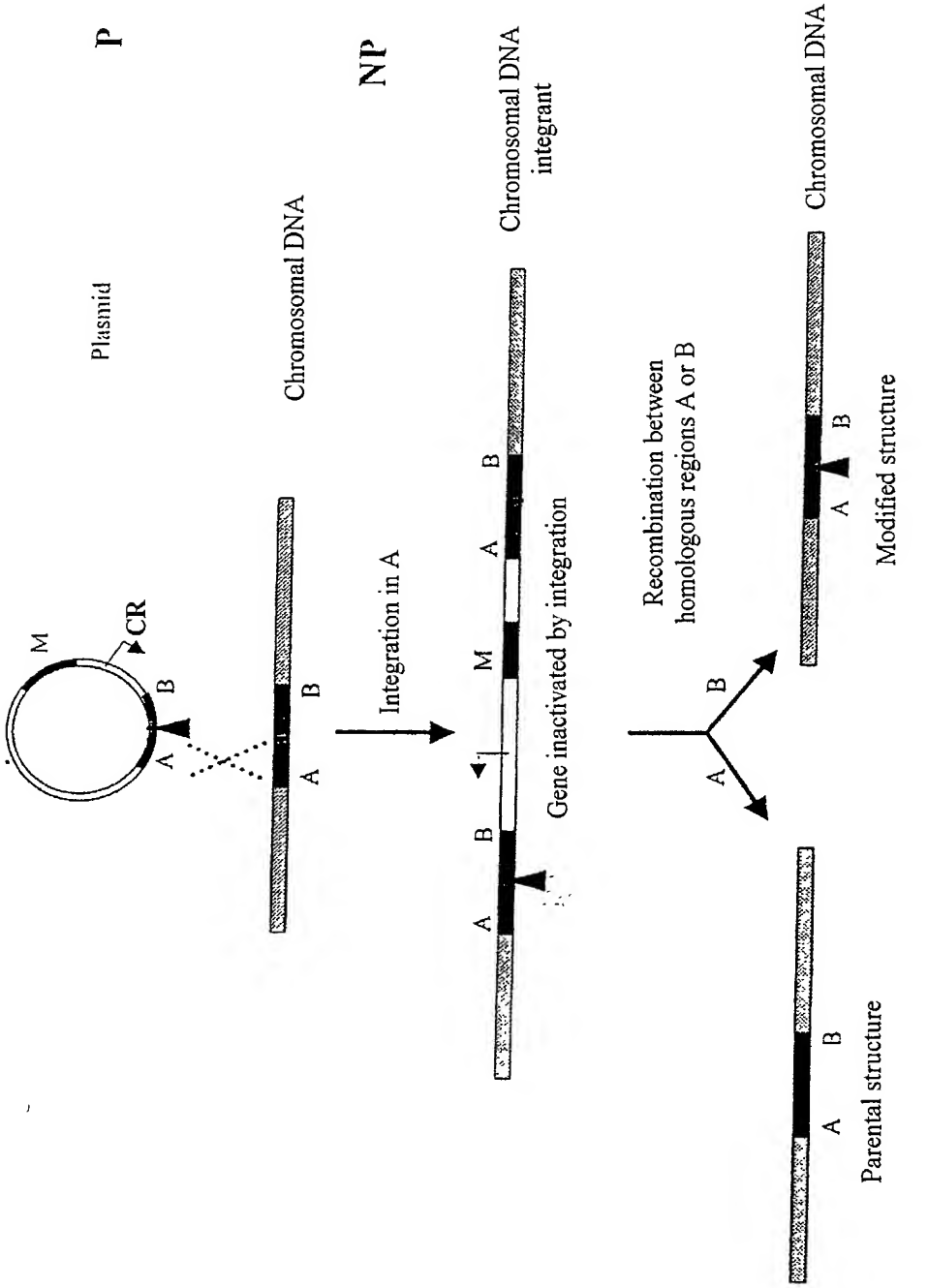
(54) Title: METHOD FOR GENETIC MODIFICATION OF LACTOBACILLUS DELBRUECKII

(54) Titre: PROCEDE DE MODIFICATION GENETIQUE DE LACTOBACILLUS DELBRUECKII

(57) Abstract: The invention concerns a method for modifying the chromosomal genetic information of *Lactobacillus delbrueckii*, using a conditional integrator plasmid. The invention also concerns integrator plasmids for use in implementing said method.

(57) Abrégé: L'invention concerne un procédé de modification de l'information génétique chromosomique de *Lactobacillus delbrueckii*, mettant en oeuvre un plasmide intégratif conditionnel. L'invention concerne également des plasmides intégratifs utilisables pour la mise en oeuvre dudit procédé.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NP
Plasmid
Chromosomal DNA
Chromosomal DNA integrant
M
CR
A
B
Integration in A
Gene inactivated by integration
Recombination between homologous regions A or B
Chromosomal DNA
Modified structure
Parental structure

Figure 1

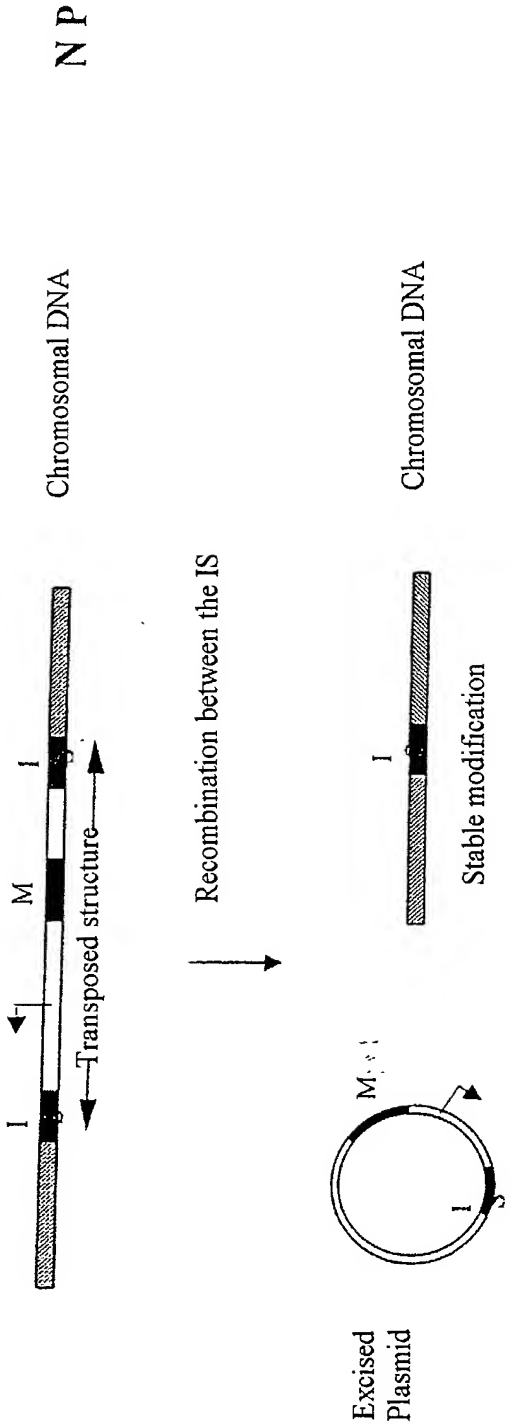
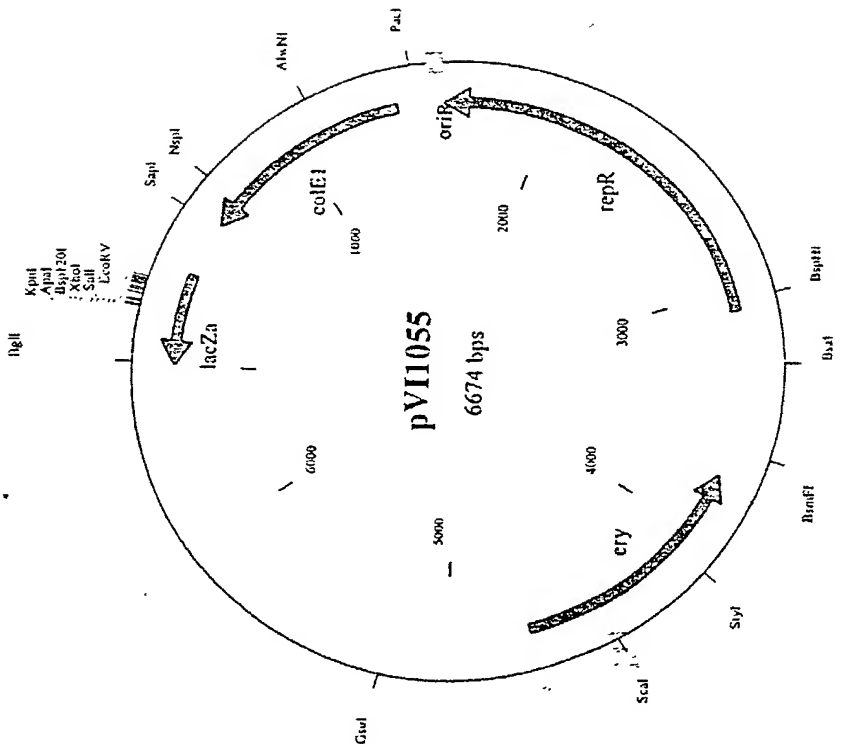

N P
Chromosomal DNA
I
M
I
Transposed structure
Recombination between the IS
Chromosomal DNA
I
Stable modification
M
I
Excised
Plasmid

Figure 2


pVI1055
6674 bps
oriR
repR
cry
colE1
lacZa
1000
2000
3000
4000
5000
6000
AlwNI
NgoMIV
SapI
NgoMI
KpnI
ApaI
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BspHI
BsrI
BsmFI
SyI
ScaI
GsuI

Figure 3

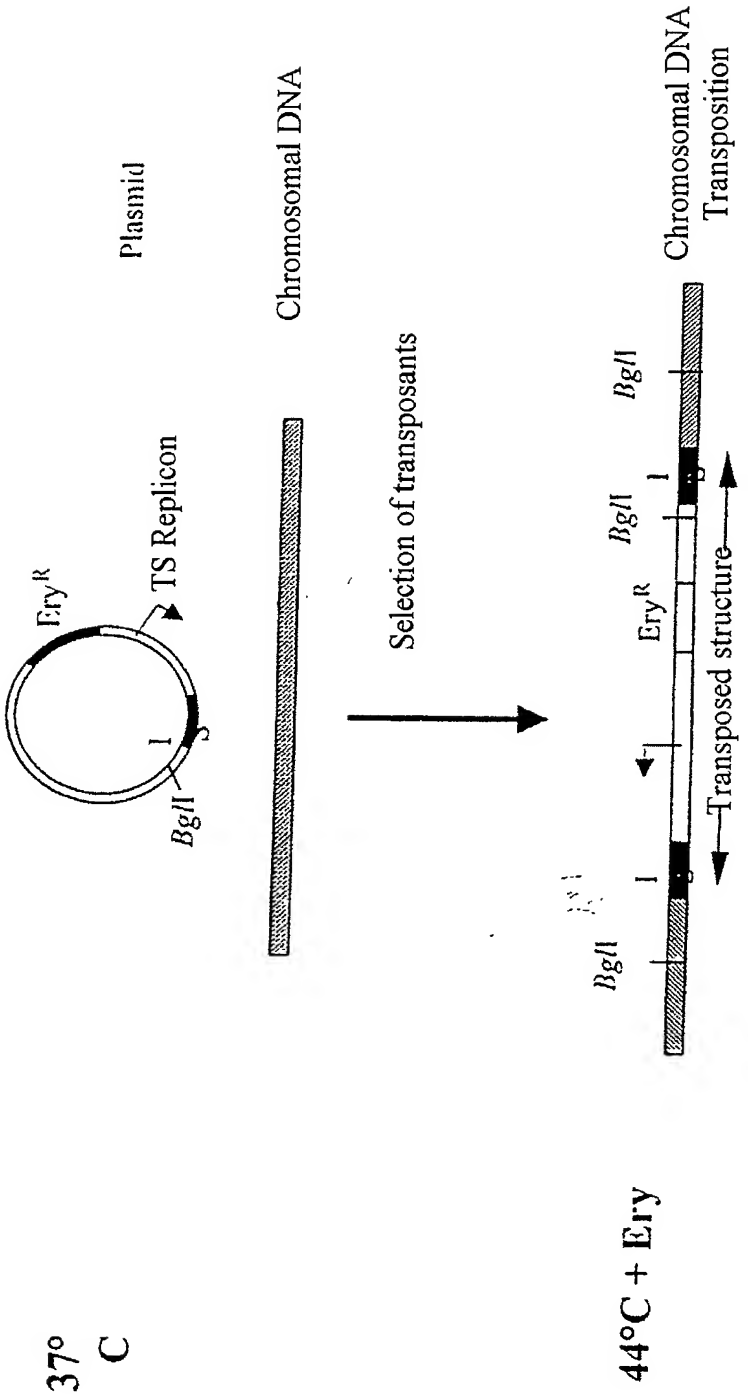
Plasmid
Chromosomal DNA
Chromosomal DNA
Transposition
Ery^R
TS Replicon
1
5
BglI
Selection of transposants
BglI
BglI
1
Ery^R
Transposed structure
BglI
1
37° C
44°C + Ery

Figure 4

COMBINED DECLARATION FOR PATENT APPLICATION AND POWER OF ATTORNEY

#4

U.S. DEPARTMENT OF COMMERCE
Patent and Trademark Office

ATTORNEY DOCKET NO. :

As a below named inventor, I hereby declare that :

My residence, post office address and citizenship are as stated below next to my name,

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled :

METHOD FOR GENETIC MODIFICATION OF *LACTOBACILLUS* DELBRUEKII

the specification of which :

is attached hereto ; or

was filed as United States application Serial No. ___10/088,293_____ on ___March 15, 2002____ and was amended on _____ (if applicable) ; or

was filed as a PCT international application Number PCT/FR00/02565 on September 15, 2000 and was amended under PCT article 19 on _____ (if applicable).

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose the U.S. Patent and Trademark Office information which is material to the patentability of claims presented in this application in accordance with Title 37, Code of Federal Regulations, § 1.56.

I hereby claim foreign priority benefits under Title 35, United States Code, §119(a)-(d) or §365(b) of any foreign application(s) for patent or inventor's certificate or §365(a) of any PCT international application(s) designating at least one country other than the United States of America listed below and have also identified below any foreign application(s) for patent or inventor's certificate or any PCT international application(s) designating at least one country other than the United States of America filed by me on the same subject matter having a filing date before that of the application(s) of which priority is claimed :

PRIOR FOREIGN APPLICATION(S) :

COUNTRY (if PCT, indicate PCT)	APPLICATION NUMBER	DATE OF FILING (day, month, year)	PRIORITY CLAIMED
FRANCE	99/11684	17 September 1999	X

Combined Declaration For Patent Application and Power of Attorney – **(Continued)**
(includes Reference to PCT International Applications)

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I hereby claim the benefits under Title 35, United States Code §119(e) of any United States provisional application(s) listed below.

U.S. PROVISIONAL APPLICATIONS

U.S. PROVISIONAL APPLICATION NO.	U.S. FILING DATE

I hereby claim the benefit under Title 35, United States Code, §120 of any United States application(s) or §365 (c) of any PCT international application(s) designating the United States of America that is/are listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in that/those prior application(s) in the manner provided by the first paragraph of Title 35, United States Code, §112, I acknowledge the duty to disclose to the U.S Patent and Trademark Office all information known to me to be material to the patentability of claims represented in this application in accordance with Title 37, Code of Federal Regulations, §1.56 which became available between the filing date of the prior application(s) and the national or PCT international filing date of this application :

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U.S. APPLICATIONS		STATUS (Check one)		
U.S. APPLICATION NO.	U.S. FILING DATE	PATENTED	PENDING	ABANDONED

POWER OF ATTORNEY: As a named inventor, I hereby appoint the registered practitioners of Morgan, Lewis & Bockius LLP included in the Customer Number provided below to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith, and direct all correspondence be addressed to that Customer Number.

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I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

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EIGHTH INVENTOR'S SIGNATURE		DATE
FULL NAME OF NINTH INVENTOR		
RESIDENCE & CITIZENSHIP		COUNTRY OF CITIZENSHIP
POST OFFICE ADDRESS		
NINTH INVENTOR'S SIGNATURE		DATE